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### Title

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### Permalink

<https://escholarship.org/uc/item/4293g8vd>

### Journal

The Journal of general physiology, 146(6)

### ISSN

0022-1295

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### Publication Date

2015-12-01

### DOI

10.1085/jgp.201511413

Peer reviewed

# Modulation of the slow/common gating of CLC channels by intracellular cadmium

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Members of the CLC family of Cl<sup>−</sup> channels and transporters are homodimeric integral membrane proteins. Two gating mechanisms control the opening and closing of Cl<sup>−</sup> channels in this family: fast gating, which regulates opening and closing of the individual pores in each subunit, and slow (or common) gating, which simultaneously controls gating of both subunits. Here, we found that intracellularly applied Cd<sup>2+</sup> reduces the current of CLC-0 because of its inhibition on the slow gating. We identified CLC-0 residues C229 and H231, located at the intracellular end of the transmembrane domain near the dimer interface, as the Cd<sup>2+</sup>-coordinating residues. The inhibition of the current of CLC-0 by Cd<sup>2+</sup> was greatly enhanced by mutation of I225W and V490W at the dimer interface. Biochemical experiments revealed that formation of a disulfide bond within this Cd<sup>2+</sup>-binding site is also affected by mutation of I225W and V490W, indicating that these two mutations alter the structure of the Cd<sup>2+</sup>-binding site. Kinetic studies showed that Cd<sup>2+</sup> inhibition appears to be state dependent, suggesting that structural rearrangements may occur in the CLC dimer interface during Cd<sup>2+</sup> modulation. Mutations of I290 and I556 of CLC-1, which correspond to I225 and V490 of CLC-0, respectively, have been shown previously to cause malfunction of CLC-1 Cl<sup>−</sup> channel by altering the common gating. Our experimental results suggest that mutations of the corresponding residues in CLC-0 change the subunit interaction and alter the slow gating of CLC-0. The effect of these mutations on modulations of slow gating of CLC channels by intracellular Cd<sup>2+</sup> likely depends on their alteration of subunit interactions.

## INTRODUCTION

Members of the CLC Cl<sup>−</sup> channel/transporter family participate in a wide spectrum of biological processes from extreme acid resistance in enteric bacteria to lysosomal acidification and membrane voltage regulation in mammalian cells (Jentsch, 2008; Jentsch et al., 2005; Stauber et al., 2012). These CLC proteins contain two identical subunits (Middleton et al., 1994, 1996; Ludewig et al., 1996), each forming a Cl<sup>−</sup>-transport pathway (Dutzler et al., 2002, 2003). In eukaryotic CLC Cl<sup>−</sup> channels, such as CLC-0 and CLC-1, the individual anion-transport pathways (or pores) are gated by the negatively charged side chain of a glutamate residue (Dutzler et al., 2003). The swing of this glutamate side chain is believed to be responsible for the “fast gating” operating in the time range of milliseconds. There is a second gating mechanism called “slow gating” (for CLC-0) or “common gating” (for CLC-1), which controls the opening and closing of both pores simultaneously with a slower kinetics (Chen, 2005; Miller, 1982; Miller and White, 1984). Although recent work has identified certain molecular determinants for the slow/common gating of CLC channels (Bennetts and Parker,

2013), the exact mechanism of this gating process is not well understood.

It has been suggested that interactions between the two CLC subunits are involved in the slow/common gating processes (Estévez and Jentsch, 2002; Bykova et al., 2006). Crystal structures of the *Escherichia coli* CLC (CLC-ec1) and a eukaryotic CLC transporter CmCLC show extensive helix packing in the subunit interface (Dutzler et al., 2002; Feng et al., 2010; Robertson et al., 2010). At this flat, nonpolar interface, hydrophobic residues such as valine, leucine, and isoleucine form most of the cross-subunit contacts (Dutzler et al., 2002; Robertson et al., 2010). Replacing two isoleucine residues, I201 and I422, at the dimer interface of CLC-ec1 (see Fig. 1 for the locations of these two residues) with tryptophan disrupts the dimeric architecture, and the I201W/I422W double mutant of CLC-ec1 exists as a monomer (Robertson et al., 2010). Coincidentally, previous work has also demonstrated that mutations of I290 and I556 of CLC-1 (which correspond to I201 and I422, respectively, in CLC-ec1) alter the common gating (Pusch et al., 1995; Kubisch et al., 1998; Saviane et al., 1999). Collectively, these results suggest that molecular

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Abbreviations used in this paper: FRET, fluorescent resonant energy transfer; HEK, human embryonic kidney; MTSES, 2-sulfonatoethyl MTS; MTSET, 2-(trimethylammonium)ethyl MTS.

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events occurring in the CLC dimer interface contribute to slow/common gating.

The research in our laboratory has revealed previously that extracellular zinc ions ( $\text{Zn}^{2+}$ ) inhibit CLC-0 by facilitating the closure of slow gate, although the  $\text{Zn}^{2+}$ -binding site for this effect is not known (Chen, 1998). In the present study, we find that intracellular transition metal cations (such as  $\text{Cd}^{2+}$ ) also inhibit the slow gating in WT CLC-0. We have identified that the  $\text{Cd}^{2+}$ -binding site responsible for this inhibition effect in CLC-0 is formed by C229 and H231 located at the dimer interface near the intracellular end of the transmembrane domain (Fig. 1). Interestingly, the  $\text{Cd}^{2+}$  inhibition potency in CLC-0 is drastically increased by I225W/V490W mutations, corresponding to the dimer interface mutations of I201W/I422W in CLC-ec1. In addition, formation of a disulfide bond between introduced cysteine residues in this binding site is also affected by mutations of I225W and V490W. These results show that a structural change of the  $\text{Cd}^{2+}$ -binding site, as reported by the alterations of  $\text{Cd}^{2+}$  modification and disulfide-bond formation, is induced by the dimer interface mutations, which also alter CLC channel's slow/common gating. These results thus suggest that a structural rearrangement at the dimer interface near the intracellular  $\text{Cd}^{2+}$ -binding site is likely involved in the slow/common gating of CLC channels.

## MATERIALS AND METHODS

### Chemicals

MTS reagents were purchased from Toronto Research Chemicals. Stock solutions of 0.3 M MTS reagents were made in distilled water and stored at  $-80^{\circ}\text{C}$ . The working solutions with required concentrations of MTS reagents were made immediately before use from the stock solution placed on ice, and were discarded if not used within 5 min. Cadmium chloride ( $\text{CdCl}_2$ ), HEPES,

EGTA, and various salts for electrophysiological recordings were from Sigma-Aldrich and Thermo Fisher Scientific.

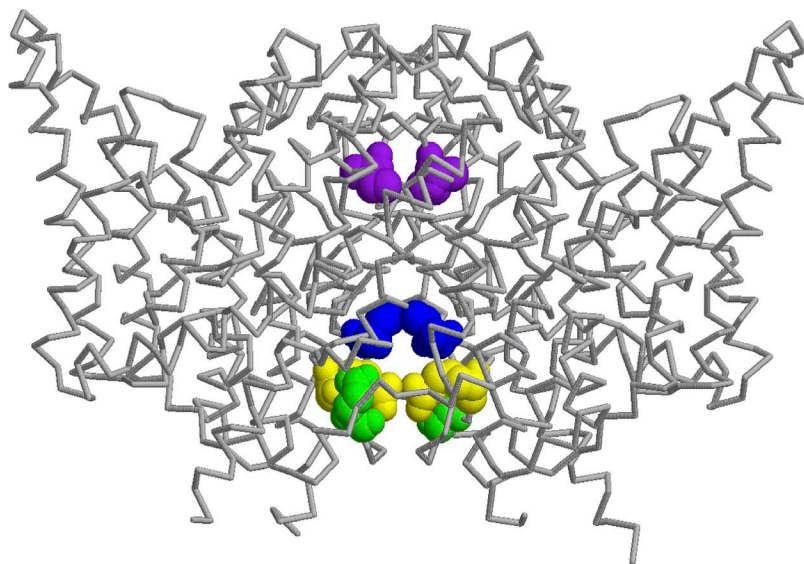
### Mutagenesis and channel expression

The CLC-0 and CLC-1 cDNAs used for electrophysiological recordings were constructed in the pcDNA3 vector without any genetic tags of fluorescent proteins. For the constructs used in biochemical experiments, a "Flag" epitope was added to the N terminus (after the first methionine) of different CLC-0 constructs subcloned in the pcDNA3 vector. Mutations were made using the QuikChange site-directed mutagenesis kit (Agilent Technologies) and were confirmed by commercial DNA sequencing services. In the fluorescent resonant energy transfer (FRET) study used to evaluate the interaction of two channel subunits (Fig. 3), the C-terminal cytoplasmic domain of CLC-0 was removed after Ser530 to make a fair comparison with CLC-ec1 because the latter lacks a cytoplasmic domain. This C terminus-truncated mutant of CLC-0, referred to as CLC0-TM, has been described previously (Bykova et al., 2006). It was constructed in the pEGFP-N3 vector, and the C terminus of the CLC0-TM sequence was tagged by a Gly-Ser linker followed by the sequence of cerulean or eYFP. The I225W/V490W mutations of CLC-0 (corresponding to I201W/I422W mutations in CLC-ec1) were made in these two CLC0-TM constructs, and were used in the FRET study. The CNG channel used as the negative control in the FRET experiment was provided by W.N. Zagotta (University of Washington, Seattle, WA), and fluorescent proteins were also tagged to the C terminus of the CNG channel.

All experiments on WT CLC-0 and its mutants were conducted in human embryonic kidney (HEK)293 cells, whereas experiments on WT and mutant CLC-1 were conducted in tsA201 cells. Transfections of cDNA into HEK293 cells or tsA201 cells followed a procedure described previously (Lin and Chen, 2003; Zhang et al., 2006, 2010; Zhang and Chen, 2009), using the lipofectamine transfection kit (Life Technologies). Experiments were performed 1–3 d after transfections.

### Electrophysiological recordings

Constructs of WT or mutant CLC channels were cotransfected with the cDNA of the green fluorescent protein (GFP). The green fluorescence was visualized via an inverted microscope (DM IRB; Leica) equipped with a GFP filter (Chroma Technology Corp.). Macroscopic currents from excised inside-out membrane patches of the transfected cells were recorded according to methods described previously (Lin and Chen, 2003; Zhang et al., 2006, 2010;



**Figure 1.** Crystal structure of CLC-ec1 as a guiding roadmap for examining the transmembrane domains of CLC-0 and CLC-1. The residues R205 (yellow) and Q207 (green) of CLC-ec1 correspond to C229 and H231 of CLC-0, or S294 and Y296 of CLC-1, respectively. Residues I201 (blue) and I422 (purple) are also depicted, which correspond to I225 and V490 of CLC-0, or I290 and I556 of CLC-1, respectively. The double mutations I201W/I422W in CLC-ec1 disrupted the dimeric interaction in this *E. coli* CLC and generated functional monomeric CLC-ec1 proteins (Robertson et al., 2010).

Zhang and Chen, 2009). Both  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  (not depicted) can inhibit WT CLC-0 from the intracellular side with roughly similar potency. However,  $\text{Zn}^{2+}$  inhibition on the I225W/V490W mutant has a slower off-rate compared with that of the  $\text{Cd}^{2+}$  inhibition. Thus, for technical convenience, we focused on the experiments using  $\text{Cd}^{2+}$ . All electrophysiological experiments were conducted using an amplifier (Axopatch 200B; Molecular Devices), and the recording traces were digitally filtered at 5 kHz, and digitized at 10 kHz using a Digidata 1440 digitizing board and pClamp10 software (Molecular Devices). The recording pipettes were fabricated from borosilicate glass capillaries (World Precision Instruments) using the pp830 pipette puller (Narishige International, Inc.). The electrodes normally had a resistance of  $\sim 1\text{--}3\text{ M}\Omega$  when filled with the pipette (extracellular) solution containing (mM): 130 NaCl, 5  $\text{MgCl}_2$ , 10 HEPES, and 1 EGTA, pH 7.4. The bath (intracellular) solution was the same as the pipette solution except that EGTA was not included in the solution containing  $\text{Cd}^{2+}$ . Because the two gating mechanisms in WT CLC-0 operate in very different time scales (Miller, 1982; Miller and White, 1984; Maduke et al., 2000; Chen, 2005), a voltage protocol containing multiple short voltage steps was used to construct the voltage-dependent activation curve (which reflects the fast-gate  $P_o$ -V curve): a prepulse at 60 mV was given before the test voltage was clamped from 60 to  $-160\text{ mV}$  for 100 ms in  $-20\text{-mV}$  voltage steps, followed by a tail voltage pulse at  $-100\text{ mV}$ . The voltage-dependent fast-gate activation curve was constructed by normalizing the initial tail current after various test voltages to that after the most depolarized test voltage. For the hyperpolarization-activated mutants such as the I225W/V490W double mutant and the mutants constructed in the I225W/V490W background, the voltage protocol included a test voltage pulse from 60 to  $-160\text{ mV}$  for 40 ms in  $-10\text{-mV}$  steps followed by a  $-100\text{-mV}$  tail pulse. The P/4 leak subtraction routine was applied to the recordings of the hyperpolarization-activated mutants because no current was detected from these mutants at the membrane voltage  $>0\text{ mV}$ .

The kinetics of the two gating mechanisms of CLC-1 (fast gating and common gating) only differs by severalfold. Separating these two gating mechanisms normally relies on two voltage protocols (referred to as protocol A and protocol B; Accardi and Pusch, 2000; Duffield et al., 2003; Bennetts et al., 2005; Tseng et al., 2007). Protocol A was used to assess the overall open probability ( $P_o$ ) of CLC-1: the product of the open probability of the fast gate ( $P_o^f$ ) and that of the common gate ( $P_o^c$ ). In brief, the membrane voltage was clamped at 0 mV and was first stepped to various test voltages from 120 to  $-120\text{ mV}$  in  $-20\text{-mV}$  steps for 200 ms before the tail voltage step at  $-100\text{ mV}$  for 100 ms. The tail current at  $-100\text{ mV}$  was fitted to a double exponential function, and the  $P_o$  was calculated by normalizing the value of the initial tail current to the maximal initial tail current obtained after the most positive test voltage. Protocol B is basically the same as protocol A except that a short (400- $\mu\text{s}$ ) but very positive (170-mV) pulse was inserted before the tail voltage step to completely open the fast gate. In this case, the normalized tail current represents  $P_o^c$  at the preceding test voltage, and  $P_o^f$  can be calculated by dividing  $P_o$  by  $P_o^c$ .

In experiments where continuous monitoring of the channel current was required, the membrane potential was clamped at 0 mV. For the depolarization-activated channels (such as WT CLC-0, C212S, and CLC-1), a voltage pulse of 60 mV for 100–200 ms followed by a negative voltage of  $-100\text{ mV}$  for 100–300 ms was given every 2 s. For the hyperpolarization-activated mutants, the 60-mV voltage step of this pulse protocol was omitted. The steady-state current at the end of the negative voltage step was measured to monitor the various effects from  $\text{Cd}^{2+}$  modulation or from modification by MTS reagents. Solution exchange was achieved using a solution exchanger (SF-77; Warner Instruments). Various concentrations of  $\text{Cd}^{2+}$  or MTS reagents were

applied to the intracellular side of the excised inside-out membrane patches.

Examining the kinetics of  $\text{Cd}^{2+}$  inhibition on the I225W/V490W mutant is challenging because monitoring the channel current requires negative voltage pulses that relieve the binding of the  $\text{Cd}^{2+}$ . We therefore designed a two-pulse protocol for studying the kinetics of  $\text{Cd}^{2+}$  inhibition. To evaluate the inhibition rate of intracellular  $\text{Cd}^{2+}$ , a  $-100\text{-mV}$  voltage pulse for 50 ms was first given to obtain the initial current in the absence of  $\text{Cd}^{2+}$ , followed by exposing the patch to various concentrations of  $\text{Cd}^{2+}$  at 0,  $-40$ , or  $-80\text{ mV}$  for various periods of time before the same pulse was given again to measure the remaining current (Fig. 12 A). Combining multiple traces from experiments using this two-pulse protocol generates the time course of the  $\text{Cd}^{2+}$  inhibition (Fig. 12 B). To measure the recovery rate of  $\text{Cd}^{2+}$  inhibition, a 50-ms voltage pulse to  $-100\text{ mV}$  was given to obtain the control current, followed by exposing the patch to 300  $\mu\text{M}$   $\text{Cd}^{2+}$  for 2 s.  $\text{Cd}^{2+}$  was then washed out for various amounts of time at 0,  $-40$ , or  $-80\text{ mV}$  before a second voltage pulse was given to assess the current. The current recorded from the second pulse was normalized to the control current before  $\text{Cd}^{2+}$  inhibition.

All solution exchanges were achieved by using a solution exchanger (SF-77; Warner Instruments). The time for the electrode tip to cross the border of two laminar flows from the solution delivery pipe of the exchanger is only 2 ms (Zhang and Chen, 2009). Including the dead time of triggering the motor that moves the solution delivery pipes, the solution exchange is usually completed within 20–50 ms.

#### FRET experiments and analyses

Experimental methods for the FRET experiments have been described previously (Bykova et al., 2006). In brief, an inverted fluorescence microscope (IX-81; Olympus) equipped with a 100-W mercury lamp was used for imaging. The intensity of the excitation light was controlled by a set of neutral density filters installed in a filter wheel, and the duration of light exposure was controlled by a computer-driven mechanical shutter (IX2-SHA; Olympus) using Metamorph software (Molecular Devices). The cell images and the images of their spectra were recorded by a CCD camera (128B; Roper Scientific). For FRET measurements, two filter cubes (Chroma Technology Corp.) were used: Cube I (excitation filter D436/20 and dichroic mirror 455dclp) was used to excite cerulean; Cube II (excitation filter HQ500/20 and dichroic mirror Q515lp) was used to excite eYFP. No emission filter was used in these cubes. Autofluorescence from untransfected cells was negligible in all experiments.

Fluorescence imaging and analysis were performed using the Metamorph software (Molecular Devices). The experiments were achieved by user-designed journals that initiated automatic collection of a bright field cell image, the fluorescence cell images (taken with 50 ms of light exposure), and finally the spectral images (800 ms of light exposure). Spectral images were generated from the fluorescence signal on the cell by passing the imaging through a slit of a spectrograph (Acton SpectraPro 2150i; Princeton Instruments; Fig. 3 A); the same slit position was applied to both the spectrum from cerulean excitation and the spectrum from eYFP excitation. Fluorescence signals from the truncated WT CLC-0 and the truncated I225W/V490W mutant can be clearly identified at the membrane (Fig. 3 A). Therefore, the edge of the spectra corresponding to the membrane fluorescent signal was used for FRET analysis. Background light spectra, collected from the blank region of the same image, were subtracted from the cerulean and the eYFP spectral signals.

FRET involves a nonradiative transfer of light-induced excitation from one fluorophore (donor) to another (acceptor) when the donor and acceptor are located within a distance of  $<100\text{ \AA}$



(Miyawaki and Tsien, 2000; Erickson et al., 2001; Zheng et al., 2002, 2003). The efficiency of the energy transfer ( $E$ ) depends on the distance,  $R$ , between the two fluorophores following the Forster equation:

$$E = (R_0)^6 / [(R_0)^6 + R^6],$$

where  $R_0$  is a spectroscopically determined distance when  $E = 0.5$  (or 50% of FRET efficiency). Our FRET experiments involved passing fluorescence signal of the cell through a slit of a spectrograph and obtaining the spectrum of the fluorescence (Fig. 3 A). The spectra obtained by using the cerulean excitation wavelength (wavelength centered at 436 nm) and that obtained by using the eYFP excitation wavelength (wavelength centered at 500 nm) were from cells transfected with constructs containing cerulean and eYFP, or from cells transfected with constructs containing eYFP only. FRET efficiency was calculated using the method and equations demonstrated in Bykova et al. (2006). The FRET analysis is illustrated in Fig. 3 B. In brief, from cells transfected with cDNA constructs containing eYFP only, ratio  $A_0$  is defined as the ratio between the fluorescence excited by light filtered by Cube I ( $F^{Y(Direct)}_{436}$ ) and the fluorescence excited by the light passing filter Cube II ( $F^{Y_{500}}$ ). Namely:

$$\text{Ratio } A_0 = F^{Y(Direct)}_{436} / F^{Y_{500}}.$$

Another ratio (Ratio A) was determined from cells cotransfected with constructs containing cerulean and eYFP. In this case, the cerulean fluorescence by direct excitation was obtained from cells expressing cerulean only, and was subtracted from the cerulean-excitation spectrum obtained from cells coexpressing cerulean

and eYFP. The resulted spectrum only contains emission from the acceptor (eYFP), which includes two components: the eYFP emission caused by FRET ( $F^{Y(FRET)}_{436}$ ) and the emission caused by direct excitation of eYFP ( $F^{Y(Direct)}_{436}$ ). Thus,

$$\text{Ratio A} = F^{Y_{436}} / F^{Y_{500}} = (F^{Y(FRET)}_{436} + F^{Y(Direct)}_{436}) / F^{Y_{500}}.$$

We defined FRET ratio (FR) as the ratio of A and  $A_0$ :

$$\text{FR} = \text{Ratio A} / \text{Ratio } A_0 = (F^{Y(FRET)}_{436} + F^{Y(Direct)}_{436}) / F^{Y(Direct)}_{436} = 1 + F^{Y(FRET)}_{436} / F^{Y(Direct)}_{436}.$$

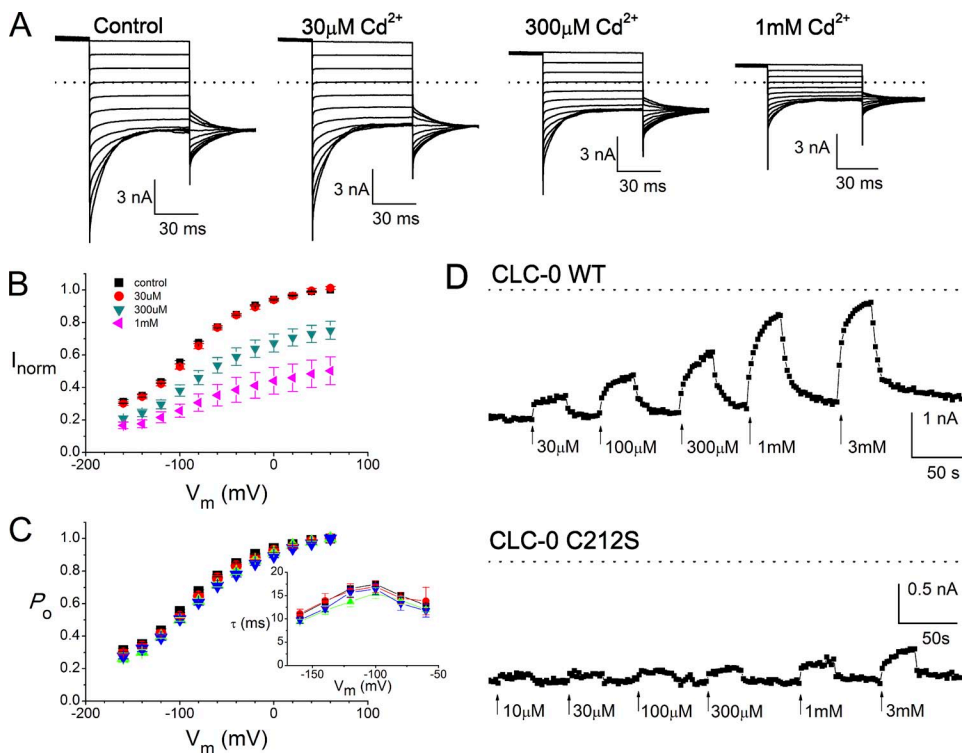
The apparent FRET efficiency ( $E_{app}$ ) is calculated according to:

$$E_{app} = (\epsilon_Y / \epsilon_C) \times (\text{FR} - 1),$$

where  $\epsilon_C$  and  $\epsilon_Y$  are molar extinction coefficients of cerulean and eYFP, respectively.

#### Biochemical experiments and Western blots

The Flag-tagged CLC-0 constructs were transfected into HEK293 cells, and the cells were harvested 2 d after transfection. The harvested cells were lysed in Dulbecco's phosphate-buffered saline (Life Technologies) containing 25 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and a protease inhibitor cocktail (Roche). The supernatant of the lysate after centrifugation was treated with or without 10  $\mu$ M copper phenanthroline before being loaded into a 7.5% SDS-PAGE gel for



**Figure 2.** Intracellular Cd<sup>2+</sup> inhibition on WT CLC-0 and the C212S mutant. (A) Original recording traces of WT CLC-0 in various intracellular [Cd<sup>2+</sup>]. The current relaxations at those hyperpolarized voltages are caused by voltage-dependent closure of the fast gate. (B) Normalized steady-state current of WT CLC-0 in different intracellular [Cd<sup>2+</sup>]: 0 (black), 30  $\mu$ M (red), 300  $\mu$ M (green), and 1 mM (purple). The initial tail currents after different test voltages in various [Cd<sup>2+</sup>] were all normalized to the maximal initial tail current in 0 [Cd<sup>2+</sup>]. (C) Fast-gate  $P_o$  as a function of voltage in the presence of 0 (black), 30  $\mu$ M (red), 100  $\mu$ M (green), and 300  $\mu$ M (blue) Cd<sup>2+</sup>, calculated by normalizing the initial tail current to the maximal tail current in the same [Cd<sup>2+</sup>]. (Inset) Current relaxation time constant of the recorded current as those shown in A, which is equal to the inverse of the sum of the opening

and the closing rate of the fast gate. The colors representing various [Cd<sup>2+</sup>] are the same as those in the  $P_o$ -V plot. (D) Comparing the effects of intracellular Cd<sup>2+</sup> between WT CLC-0 and the C212S mutant. A test pulse of 60 mV followed by a tail voltage at -100 mV was given every 2 s to monitor the Cd<sup>2+</sup> inhibition. Current was measured at the end of the tailed pulse when the current reached the steady state. Various [Cd<sup>2+</sup>] were applied at the time indicated by the arrows. Dash line represents zero-current level.

electrophoresis. The proteins were then transferred to nitrocellulose papers and detected by anti-Flag antibody (M2 antibody; Sigma-Aldrich).

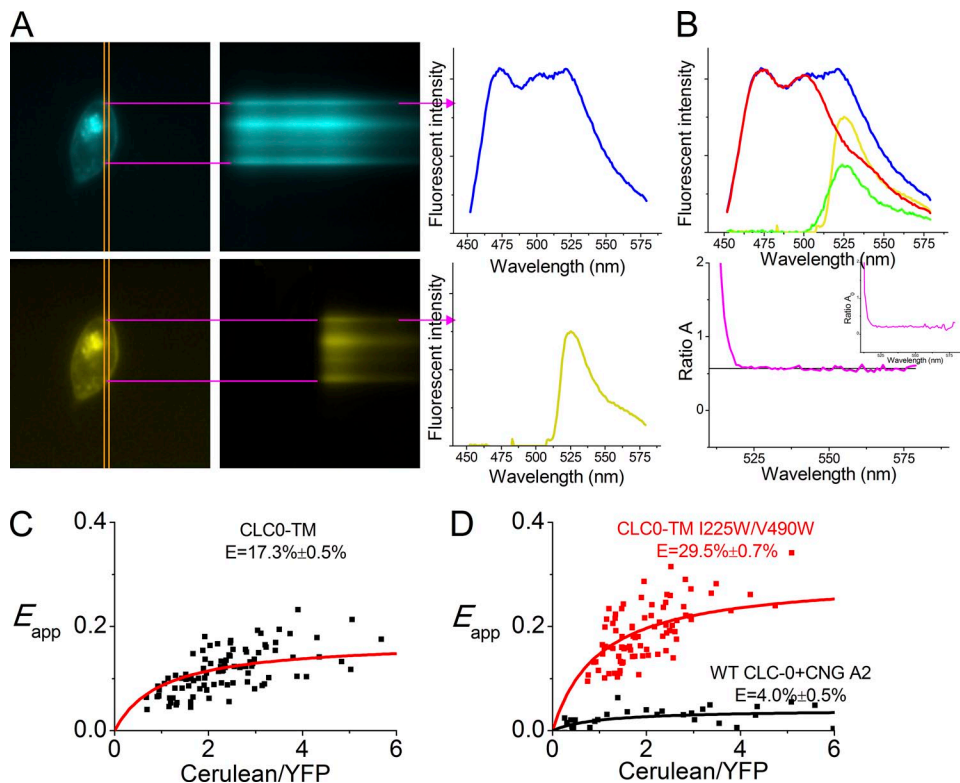
## RESULTS

Similar to the extracellular effect of  $\text{Zn}^{2+}$  on CLC-0 (Chen, 1998), intracellular  $\text{Cd}^{2+}$  can also inhibit the channel, although much higher concentrations, at least tens of micromolars, are required to generate inhibition (Fig. 2, A and B). The  $P_o$ -V curve and the kinetics of the fast gating of CLC-0 appear not to be affected by intracellular  $\text{Cd}^{2+}$  at a concentration range that significantly inhibits the channel (Fig. 2 C). Furthermore, the inhibition of the channel by  $\text{Cd}^{2+}$  is largely suppressed by the C212S mutation (Fig. 2 D), which prevents the slow gate of CLC-0 from closing (Lin et al., 1999). Collectively, these results suggest that intracellularly applied  $\text{Cd}^{2+}$  inhibits CLC-0 by modulating the slow gating.

The hypothesis that the intracellular  $\text{Cd}^{2+}$  inhibition of CLC-0 is mediated by modulating the channel's slow gating is consistent with the findings that the inhibition and recovery processes are slow (see Fig. 2 D, top). The slow kinetics of the inhibition rendered a precise characterization of the  $\text{Cd}^{2+}$  effect technically challenging because of relatively short durations of stable excised

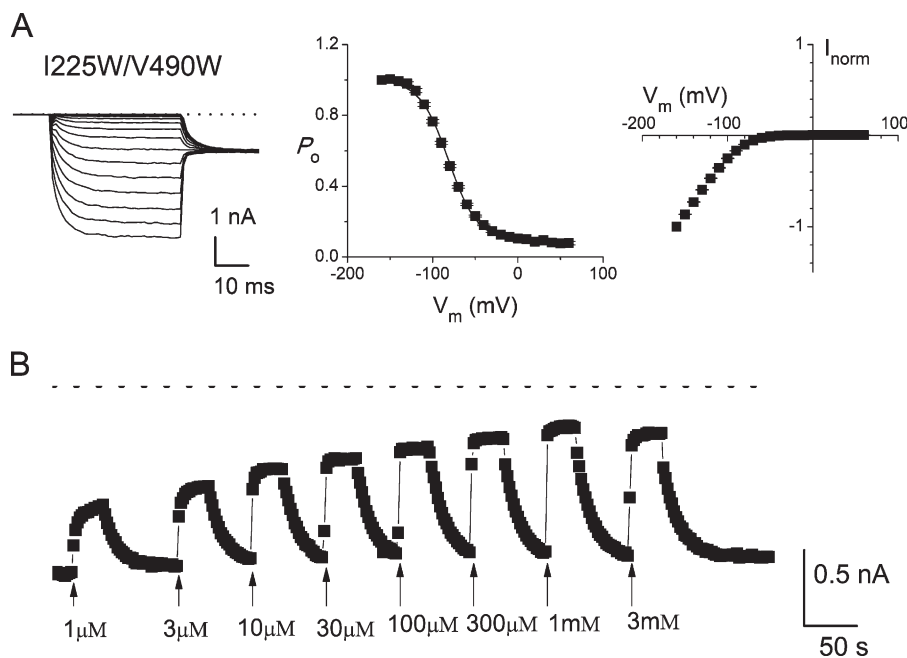
inside-out patch recordings of WT CLC-0. The problem was circumvented by an accidental finding that the mutations of I225W and V490W accelerated the inhibition kinetics. Mutations of the corresponding residues in CLC-ec1 (I201W/I422W) have been shown previously to disrupt the subunit interaction, resulting in functional monomeric CLC-ec1 proteins (Robertson et al., 2010). However, the I225W/V490W mutant of CLC-0 remains as a dimer when expressed in HEK293 cells, as shown by the FRET experiment in which the FRET efficiency between a FRET donor and a FRET acceptor attached to the I225W/V490W mutant is no less than that observed in the WT construct (Fig. 3, C and D). Nevertheless, the voltage dependence of the opening of the I225W/V490W mutant channel is opposite to that of the WT channel: the mutant channel's open probability ( $P_o$ ) increases as the membrane voltage hyperpolarizes (Fig. 4 A). Moreover,  $\text{Cd}^{2+}$  inhibition is now faster and more potent: even 1  $\mu\text{M}$   $\text{Cd}^{2+}$  significantly inhibits the current (Fig. 4 B).

A full characterization of the  $\text{Cd}^{2+}$  sensitivity of I225W/V490W is shown in Fig. 5 A, in which the currents of the mutant channel in excised membrane patches were recorded in various  $\text{Cd}^{2+}$  concentrations ( $[\text{Cd}^{2+}]$ ). We noticed that  $\text{Cd}^{2+}$  appears to have two distinct effects. First, the voltage-dependent steady-state activation



**Figure 3.** FRET experiments of the cerulean (C)- and eYFP (Y)-tagged CLC0-TM. (A) Fluorescence images of a cell expressing C- and Y-tagged CLC0-TM with I225W/V490W mutations from C (top left) and Y excitation (bottom left). The corresponding spectroscopic images (middle) were obtained by passing the signal through a slit (vertical orange lines). The emission spectra (right) were obtained from the upper edge of the spectroscopic images (arrows). (B; top) Emission spectra from the cell in A by C excitation (blue trace) and Y excitation (yellow trace). Red trace is the standard C emission spectrum from C excitation. Green trace was obtained by subtracting the red trace from the blue trace. (Bottom) Ratio A and Ratio A<sub>0</sub> (inset) are plotted against wavelength. (C) Apparent FRET efficiency ( $E_{app}$ ) of the C- and Y-tagged CLC0-TM against the C/Y intensity ratio. Each point represents the  $E_{app}$  from a cell. (D) Apparent FRET

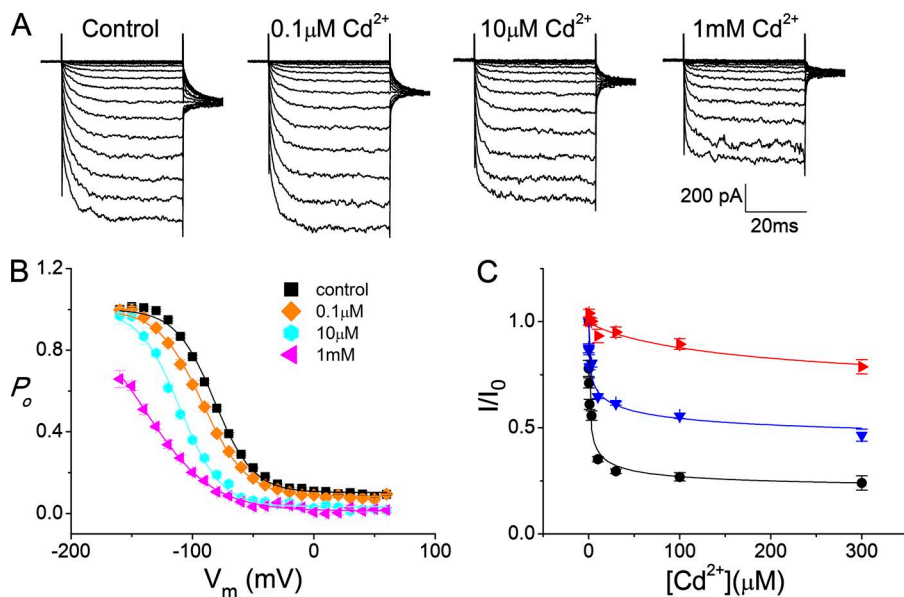
efficiency of the C- and Y-tagged CLC0-TM with I225W/V490W mutations (red dots). Black dots were from a control experiment in which cells were cotransfected with WT CLC-0 and the subunit A2 of the rat olfactory CNG channel. Data points in C and D are fitted (solid curves) to Eqs. 3 and 4 of Bykova et al. (2006). In both C and D, significant FRET efficiency ( $\sim 18\%$  for WT and  $\sim 30\%$  for I225W/V490W) was observed compared with the control experiment.



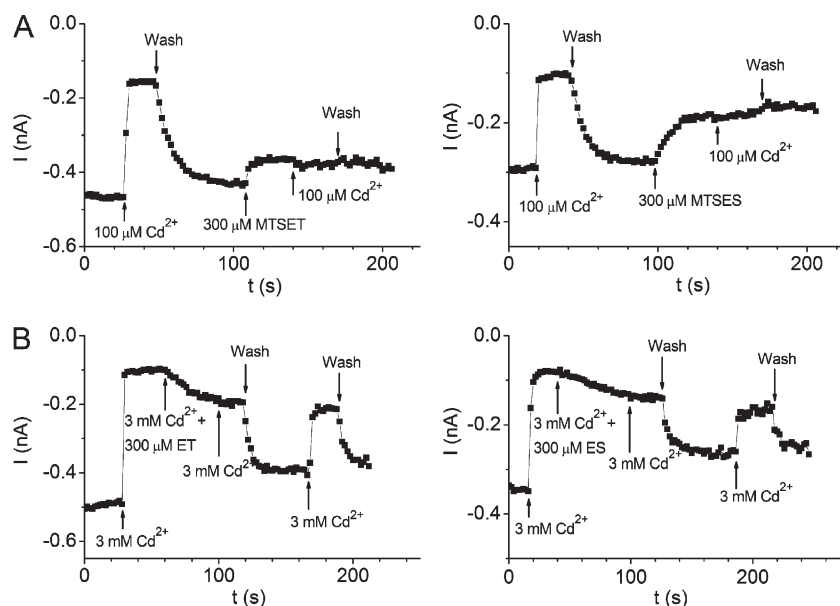
**Figure 4.** Intracellular  $Cd^{2+}$  inhibition of the I225W/V490W double mutant of CLC-0. (A) Original recording traces (left), steady-state voltage-dependent activation curve (middle), and the normalized steady-state I-V curve (right) of the I225W/V490W mutant. Data were from recording traces with p/4 leak subtraction. The dotted line in the left panel represents the zero-current level. (B) Effects of various intracellular  $[Cd^{2+}]$  on the steady-state current of the I225W/V490W mutant. A test pulse of  $-100$  mV was given every 2 s, and the current was measured at the end of the pulse to monitor the  $Cd^{2+}$  inhibition. Dotted line represents the zero-current level.  $Cd^{2+}$  of various concentrations was applied as indicated by the arrows.

curve of this mutant shifts to more hyperpolarized voltages in low  $[Cd^{2+}]$  ( $<30 \mu$ M). Second, higher  $[Cd^{2+}]$  (such as 1 mM) reduces the maximal level of the channel activation (Fig. 5 B). Because of the dual effects of  $Cd^{2+}$ , the dose-response curve of the  $Cd^{2+}$  inhibition throughout the entire  $Cd^{2+}$  concentration range cannot be fitted to a single Langmuir function. We thus focused on the inhibition by low  $[Cd^{2+}]$  (up to 300  $\mu$ M), and normalized the inhibition effect to that induced by 1 mM  $Cd^{2+}$ . The values of  $IC_{50}$  so obtained were 1, 7, and 204  $\mu$ M at  $-80$ ,  $-120$ , and  $-160$  mV, respectively (Fig. 5 C). Therefore, the inhibition of I225W/V490W by  $Cd^{2+}$  is voltage dependent; the apparent affinity is decreased with more hyperpolarized membrane potentials.

To understand the structural basis of the  $Cd^{2+}$  inhibition, we searched for the amino acid residues involved in forming the  $Cd^{2+}$ -binding site. We first tested if cysteine-modifying reagents, such as MTS compounds, could affect  $Cd^{2+}$  inhibition because binding of transition metal ions in proteins frequently involves cysteine (Rulíšek and Vondrášek, 1998). Fig. 6 A shows that the  $Cd^{2+}$  inhibition of the I225W/V490W current, monitored by a 50-ms voltage pulse from 0 to  $-100$  mV every 2 s, was indeed abolished after the patch was treated with intracellularly applied 2-(trimethylammonium)ethyl MTS (MTSET; Fig. 6 A, left) or 2-sulfonatoethyl MTS (MTSES) (Fig. 6 A, right). Consistently, the presence of intracellular  $Cd^{2+}$  reduced the effect of MTSET or



**Figure 5.** Steady-state inhibition of the I225W/V490W mutant of CLC-0 by intracellular  $Cd^{2+}$ . (A) Original recording traces in the absence of  $Cd^{2+}$  (control) and in the presence of various  $[Cd^{2+}]$ . (B) Voltage-dependent activation curves of I225W/V490W in 0 (control) and various  $[Cd^{2+}]$  based on the experiments similar to those shown in A. The initial tail current was normalized to that obtained after the test pulse of  $-160$  mV in the absence of  $Cd^{2+}$ . Notice that the maximal  $P_o$  of I225W/V490W is reduced at 1 mM of  $Cd^{2+}$ . (C) Dose-response curves of the  $Cd^{2+}$  inhibition of I225W/V490W at different voltages (black,  $-80$  mV; blue,  $-120$  mV; red,  $-160$  mV). Data points were fitted to a Langmuir equation with an apparent affinity of 1  $\mu$ M ( $-80$  mV), 7  $\mu$ M ( $-120$  mV), and 204  $\mu$ M ( $-160$  mV), respectively.

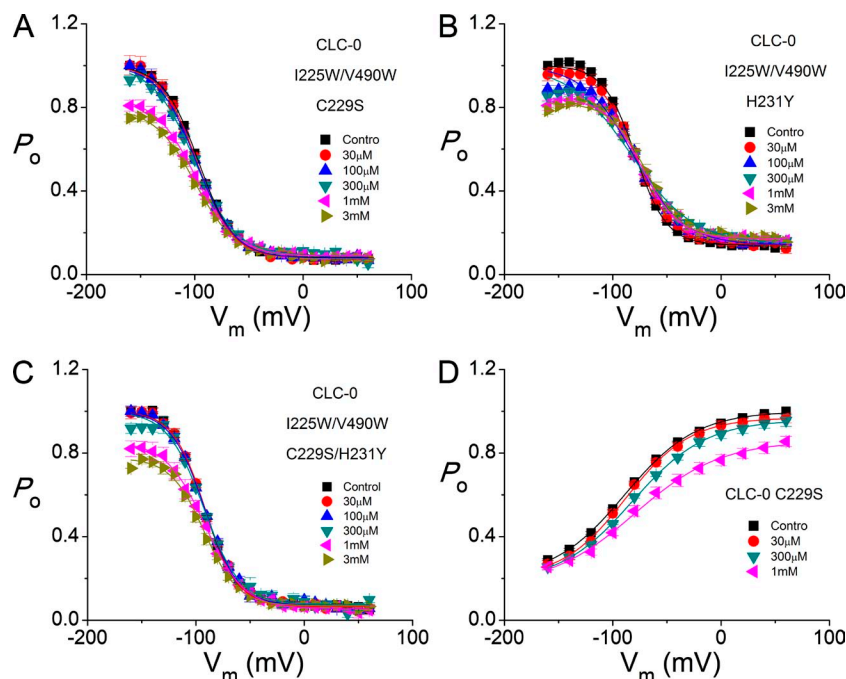


**Figure 6.** Competition between Cd<sup>2+</sup> binding and the MTS modification in the I225W/V490W mutant of CLC-0. Current was monitored by a 40-ms test pulse of  $-100$  mV every 2 s. MTS reagents and Cd<sup>2+</sup> were applied or washed out at the time points indicated by arrows. (A) Modification of the I225W/V490W mutant with intracellular MTSET (left) or MTSES (right) suppresses the Cd<sup>2+</sup> inhibition effect. (B) Cd<sup>2+</sup> binding prevents the modification by MTSET (left) or MTSES (right) on the I225W/V490W mutant.

MTSES (Fig. 6 B), indicating that at least one cysteine forms part of the binding site, so that Cd<sup>2+</sup> binding would protect the cysteine from MTS modifications.

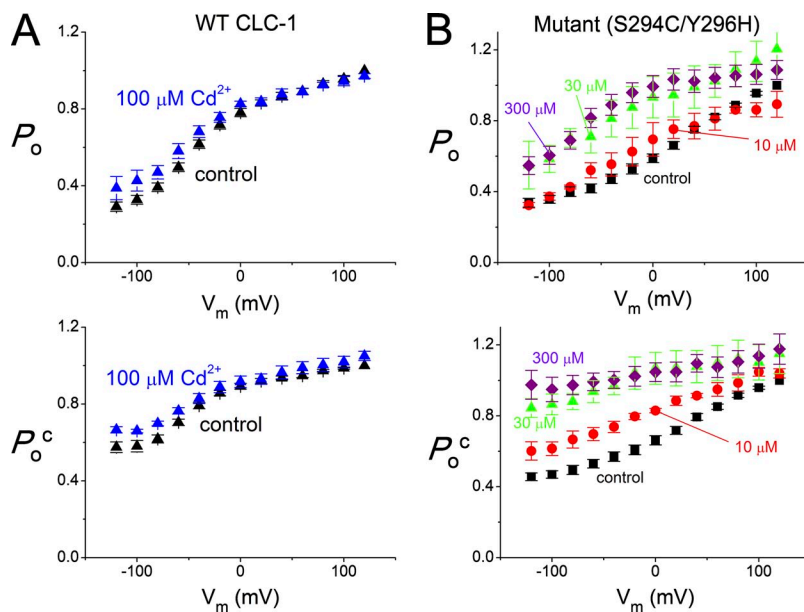
Among the 12 endogenous cysteines in CLC-0, we have shown previously that C229, which is located at the dimer interface near the intracellular end of the transmembrane domain, can be modified by intracellular MTS reagents (Zhang et al., 2010). Interestingly, a histidine residue, H231, is located nearby, raising the possibility that it can act with C229 to coordinate Cd<sup>2+</sup> as a bidentate ligand (Puljung and Zagotta, 2011). We thus tested whether C229 and H231 could both be involved in the formation of the Cd<sup>2+</sup>-binding site. Mutating

C229 (C229S), H231 (H231Y), or both (C229S/H231Y) in the background of I225W/V490W indeed suppressed the ability of Cd<sup>2+</sup> to shift the steady-state activation curve (Fig. 7, A–C, respectively). When the C229S mutation was made in the WT CLC-0, the Cd<sup>2+</sup> inhibition effect was also significantly reduced (Fig. 7 D). Likewise, the mutation H231Y also reduced the sensitivity of Cd<sup>2+</sup> inhibition on WT CLC-0 (not depicted). However, the reduction of maximal channel activity by high [Cd<sup>2+</sup>] still existed in these mutants (Fig. 7, A–D), suggesting that it is mediated by a different mechanism. Our results thus demonstrate that C229 and H231 form the high affinity Cd<sup>2+</sup>-binding site, where binding of Cd<sup>2+</sup>



**Figure 7.** Mutations of C229 and/or H231 suppress the Cd<sup>2+</sup> effect in shifting the  $P_o$ -V curve of I225W/V490W and WT CLC-0. (A–C) Steady-state  $P_o$ -V curves of I225W/V490W with the C229S single mutation (A), the H231Y single mutation (B), or the C229S/H231Y double mutations (C) in the presence of various [Cd<sup>2+</sup>]. Notice that the shift of the  $P_o$ -V curve by Cd<sup>2+</sup> is abolished, whereas the inhibition of the maximal  $P_o$  at high concentrations of Cd<sup>2+</sup> is still present. (D) Steady-state  $P_o$ -V curves of the C229S mutant on the WT background in different [Cd<sup>2+</sup>].

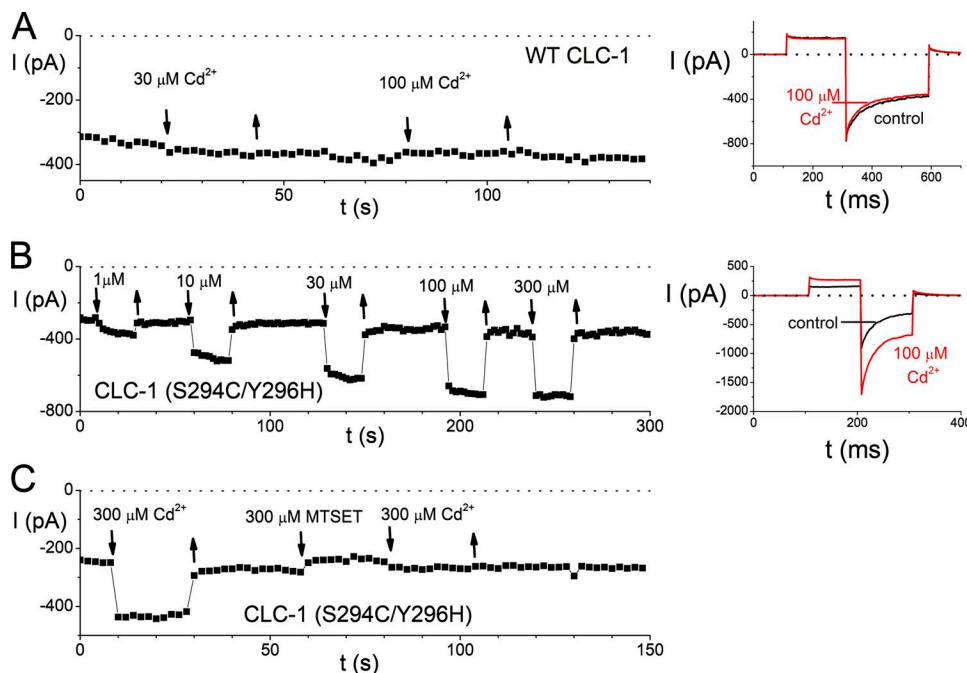




**Figure 8.** Introducing a cysteine and a histidine residue to positions in CLC-1 that correspond to C229 and H231 of CLC-0 enables a modulation of CLC-1 by micromolar  $[\text{Cd}^{2+}]$ . (A) The overall channel open probability ( $P_o$ ; top) and the common-gate open probability ( $P_o^c$ ; bottom) of WT CLC-1 as a function of voltage in the absence (control, black) and presence of 100  $\mu$ M  $\text{Cd}^{2+}$  (blue). (B) The  $P_o$ - $V$  and  $P_o^c$ - $V$  curves of the S294C/Y296H mutant of CLC-1 in various intracellular  $[\text{Cd}^{2+}]$ . C229 and H231 of CLC-0 are not conserved in CLC-1, and as expected, 100  $\mu$ M  $\text{Cd}^{2+}$  has nearly no effect on WT CLC-1 (Fahlke et al., 1998). However, 10  $\mu$ M  $\text{Cd}^{2+}$  significantly increases the open probability of the common gate ( $P_o^c$ ) of the CLC-1 mutant S294C/Y296H. Notice the opposite effect of  $\text{Cd}^{2+}$  on the open probability of CLC-1 (potentiation) compared with that of CLC-0 (inhibition).

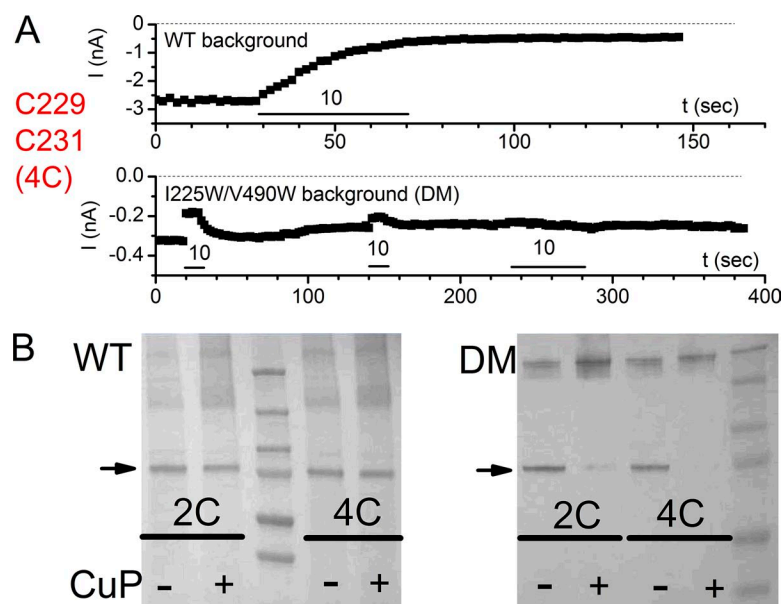
shifts the steady-state activation curve of I225W/V490W. It is likely that C229 and H231 from both CLC-0 subunits together coordinate  $\text{Cd}^{2+}$  binding, as these residues are located near the dimer interface and therefore are close to each other (see Fig. 1). The conclusion that C229 and H231 form the  $\text{Cd}^{2+}$ -binding site is further supported by experiments from CLC-1. These two residues are not conserved in CLC-1, which is not sensitive to intracellular  $\text{Cd}^{2+}$  modulation (Figs. 8 A and 9 A).

When cysteine and histidine were introduced to the corresponding positions in CLC-1 (S294C/Y296H), a  $\text{Cd}^{2+}$ -binding site was created, and the activation of CLC-1 could be modulated by intracellular  $\text{Cd}^{2+}$  (Figs. 8 B and 9, A and B). Like the MTS modification of the endogenous cysteine (C229) in CLC-0, modification of the introduced cysteine (S294C) abolished  $\text{Cd}^{2+}$  inhibition on CLC-1 containing the engineered  $\text{Cd}^{2+}$ -binding site (Fig. 9 C).



**Figure 9.** Suppressing the  $\text{Cd}^{2+}$  effect in the S294C/Y296H mutant of CLC-1 by intracellular MTS reagents. (A) Micromolar concentrations of intracellular  $\text{Cd}^{2+}$  have little effect on the WT CLC-1. The current was monitored continuously by a pulse of 60 mV followed by a voltage step at  $-100$  mV. Intracellular  $\text{Cd}^{2+}$  was applied (downward arrows) and washed out (upward arrows) as indicated. Inset shown on the right panel is a comparison of two original recording traces taken before (black) and after (red) the application of 100  $\mu$ M  $\text{Cd}^{2+}$ . (B) Intracellular  $\text{Cd}^{2+}$  potentiates the current of the S294C/Y296H double mutant of CLC-1. Inset on the right is a comparison of two original recording traces taken before (black) and after (red) 100  $\mu$ M  $[\text{Cd}^{2+}]$ . Notice that the current deactivation kinetics at the hyperpolarization voltage caused by the closure of the fast gate is not altered by  $\text{Cd}^{2+}$ , indicating that the effect is not on the fast gating of the channel. (C) Potentiation of the S294C/Y296H mutant by intracellular  $\text{Cd}^{2+}$  is abolished after modifying the channel with intracellular MTSET, indicating that the introduced cysteine and histidine at the positions of S294 and Y296 form a  $\text{Cd}^{2+}$ -binding site in CLC-1 as well.

hyperpolarization voltage caused by the closure of the fast gate is not altered by  $\text{Cd}^{2+}$ , indicating that the effect is not on the fast gating of the channel. (C) Potentiation of the S294C/Y296H mutant by intracellular  $\text{Cd}^{2+}$  is abolished after modifying the channel with intracellular MTSET, indicating that the introduced cysteine and histidine at the positions of S294 and Y296 form a  $\text{Cd}^{2+}$ -binding site in CLC-1 as well.

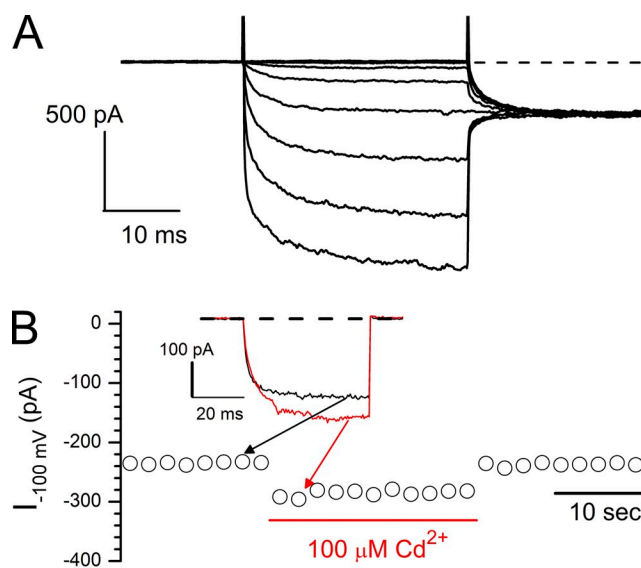


**Figure 10.** Disulfide bond linking two CLC subunits. (A) Inhibition of CLC-0 by  $\text{Cd}^{2+}$  binding to the binding site containing four cysteines in the WT CLC-0 background (top) and the dimer interface mutant background (DM; bottom). Excised inside-out patch recordings. The voltage pulse (60 mV followed by  $-100$  mV) for monitoring the current was given every 2 s, and the current at the end of the  $-100$ -mV voltage was monitored. Dotted lines, zero current level. Horizontal bars indicate the application of 10  $\mu$ M  $\text{Cd}^{2+}$ . (B) I229W/V490W dimer interface mutations (DM) facilitate disulfide bond formation in copper phenanthroline (CuP) between the two C231 residues. “4C” indicates that all four positions at positions 229 and 231 from two subunits were cysteine, whereas “2C” contained cysteine only at position 231 but not at position 229 (C229S/H231C). Arrows indicate the monomer of CLC-0, which disappeared likely because of a disulfide bond formation between the two C231 residues.

Because binding sites for transition metal cations can be formed by four cysteine residues (Yellen et al., 1994), we tested the effect of  $\text{Cd}^{2+}$  on CLC-0 with the H231C mutation. Such a CLC-0 construct contains cysteine at all four positions of 229 and 231 from two subunits (and thus will be called “4C” mutant). In the WT background, the 4C mutant becomes very sensitive to  $\text{Cd}^{2+}$ , and the  $\text{Cd}^{2+}$  inhibition is nearly irreversible (Fig. 10 A, top).  $\text{Cd}^{2+}$  inhibition of the 4C mutant with I225W/V490W background mutations, however, is very different; the inhibition can be quickly washed out, but with the time of patch excision the channel is less and less sensitive to  $\text{Cd}^{2+}$  (Fig. 10 A, bottom). We suspected that this time-dependent reduction of  $\text{Cd}^{2+}$  sensitivity might result from the formation of a disulfide bond among these four cysteine residues after patch excision, which could change the oxidation/reduction condition of the channel (Zhang et al., 2008). Indeed, Western blots shown in Fig. 10 B demonstrate that the monomeric band of the 4C mutant in the I225W/V490W background disappeared after oxidation (Fig. 10 B, right). On the other hand, the majority of the 4C mutant in the WT CLC-0 background still remained as monomers in the SDS gel after copper phenanthroline treatment (Fig. 10 B, left). Similar results were observed in a “2C” mutant in which cysteine was present only at position 231 but not at position 229 (Fig. 10 B), suggesting that it is the two cysteines at position 231 that form a disulfide bond. Thus, through the differences in  $\text{Cd}^{2+}$  inhibition and disulfide bond formation, we demonstrate that like the WT  $\text{Cd}^{2+}$ -binding site, the structure of the mutant  $\text{Cd}^{2+}$ -binding site with four cysteines is changed by the I225W/V490W mutations.

Because C229 and H231 are located near the intracellular end of the transmembrane domain and interactions

between the transmembrane domain and the cytoplasmic domain have been demonstrated in CLC molecules (Feng et al., 2010; Bennetts and Parker, 2013), the cytoplasmic domain of CLC-0 may influence  $\text{Cd}^{2+}$  inhibition. We thus tested the intracellular  $\text{Cd}^{2+}$  effect on a CLC-0



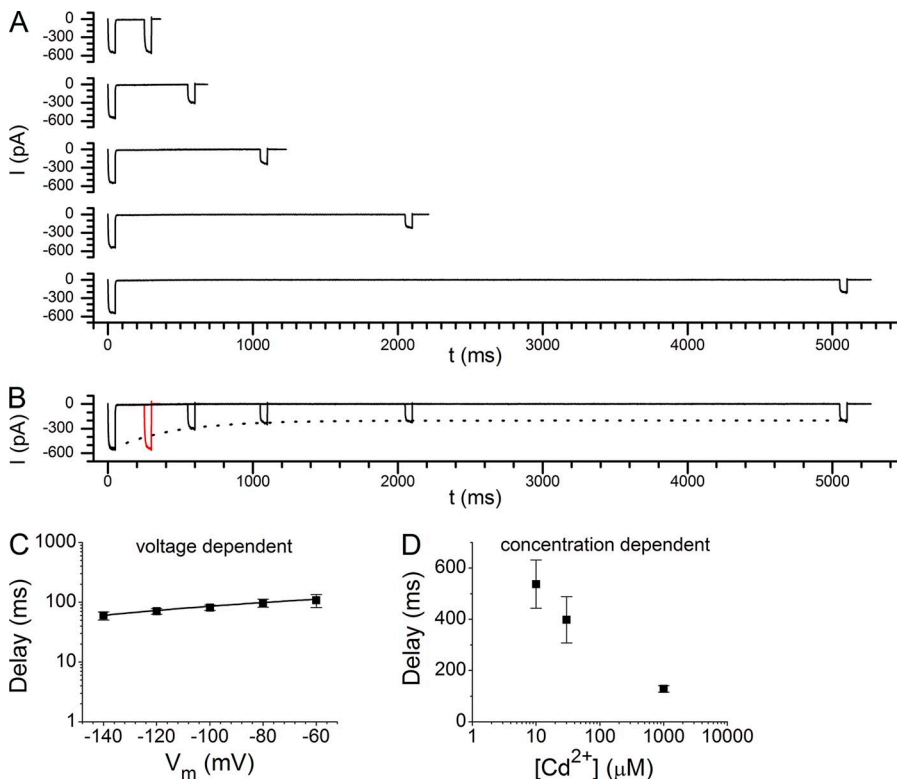
**Figure 11.** Effects of intracellular  $\text{Cd}^{2+}$  on the C terminus-truncated CLC-0 (CLC0-TM). (A) Current of the eYFP-tagged CLC0-TM activated by a voltage family from 60 to  $-160$  mV in a  $-20$ -mV voltage step. Excised inside-out patch recordings. Tail voltage was  $-100$  mV. (B) Effects of intracellular  $\text{Cd}^{2+}$  on the current of CLC0-TM monitored by continuously applying a voltage pulse of  $-100$  mV (40 ms) every 2 s. Currents at the end of the  $-100$ -mV voltage step are plotted against time. 100  $\mu$ M  $\text{Cd}^{2+}$  was applied as indicated by the horizontal red line. (Inset) Comparison of the recording traces before and after  $\text{Cd}^{2+}$  application. Dashed line indicates the zero-current level.

mutant in which the C-terminal region of the channel after residue 530 is replaced with a fluorescent protein. As reported previously (Bykova et al., 2006), this CLC-0 mutant (abbreviated as “CLC0-TM”) generates hyperpolarization-activated currents (Fig. 11 A). Interestingly,  $\text{Cd}^{2+}$  did not inhibit but potentiated this mutant (Fig. 11 B), suggesting that the cytoplasmic domain of the CLC channel may also be involved in the intracellular  $\text{Cd}^{2+}$  modulation, likely through its interaction with the transmembrane domain.

To gain further insights into the  $\text{Cd}^{2+}$  modulation mechanism, we studied the kinetics of  $\text{Cd}^{2+}$  inhibition on I225W/V490W of CLC-0, taking advantage of the faster kinetics of  $\text{Cd}^{2+}$  inhibition in this mutant. Fig. 12 A illustrates a two-pulse protocol used to examine the rate of  $\text{Cd}^{2+}$  inhibition by measuring the current of the I225W/V490W mutant elicited by a  $-100\text{-mV}$  voltage pulse before (first pulse) and after the patch was exposed to  $\text{Cd}^{2+}$  for a certain amount of time (second pulse). Combining multiple recording traces from the same patch generates the time course of  $\text{Cd}^{2+}$  inhibition (Fig. 12 B). It is worth noticing that there appears to be a delay of the current inhibition because the current recorded after applying  $\text{Cd}^{2+}$  for 0.2 s is almost the same as that before the  $\text{Cd}^{2+}$  application. This delay has little voltage dependence (Fig. 12 C), but as  $[\text{Cd}^{2+}]$  is increased, the delay is shortened (Fig. 12 D). To analyze the kinetics of  $\text{Cd}^{2+}$  inhibition like that shown in Fig. 12 B, we normalized the current induced by the second voltage pulse to the current before  $\text{Cd}^{2+}$

application, and plotted the time course of the current inhibition by  $\text{Cd}^{2+}$  (Fig. 13 A). Fitting the  $\text{Cd}^{2+}$  inhibition time course with an exponential function gives the inhibition time constant ( $\tau_{\text{on}}$ ), the inverse of which ( $1/\tau_{\text{on}}$ ) is defined as the apparent  $\text{Cd}^{2+}$  inhibition rate (or apparent on rate),  $k_{\text{on}}$ . The observation that  $k_{\text{on}}$  is a saturable function of  $[\text{Cd}^{2+}]$  (Fig. 13 B) suggests that  $\text{Cd}^{2+}$  inhibition is not a simple bimolecular reaction involving only  $\text{Cd}^{2+}$  binding. These results suggest that the binding of  $\text{Cd}^{2+}$  to the open-state channel may not immediately close the channel, and the delay in current inhibition upon  $\text{Cd}^{2+}$  application could result from the conformational change between the open and the closed state of this dimer interface mutant.

We have also measured the rate of current recovery after  $\text{Cd}^{2+}$  washout (apparent off-rate, or  $k_{\text{off}}$ ) using a similar two-pulse protocol. The current recovery process was well described by a single-exponential function (recording traces not depicted). These kinetic measurements of  $\text{Cd}^{2+}$  inhibition and recovery were conducted at multiple holding voltages (Fig. 13, C and D). The values of the apparent  $\tau_{\text{on}}$  (in  $30\text{ }\mu\text{M}$   $\text{Cd}^{2+}$ ) and  $\tau_{\text{off}}$  at 0,  $-40$ , and  $-80\text{ mV}$  are compared in Fig. 13 E. Although  $\tau_{\text{on}}$  is insensitive to the change of membrane potential, the value of  $\tau_{\text{off}}$  decreases as membrane potential becomes more negative. Therefore, the lower apparent affinity of  $\text{Cd}^{2+}$  inhibition at more negative voltages on the I225W/V490W mutant results mostly from a faster off-rate by membrane hyperpolarization. Collectively, these results suggest a state-dependent



**Figure 12.** Rate of  $\text{Cd}^{2+}$  inhibition (on rate) on the I225W/V490W mutant of CLC-0. (A) Original recording traces from one patch using the two-pulse (to  $-100\text{ mV}$ ) protocol described in Materials and methods.  $[\text{Cd}^{2+}] = 100\text{ }\mu\text{M}$ .  $V_{\text{hold}} = -40\text{ mV}$ . (B) Combining the recording traces from A shows the time course of  $\text{Cd}^{2+}$  inhibition. Dotted curve is the single-exponential fit of the current amplitude elicited at various time spots except the one at 0.2 s (the second pulse). Notice the delay of  $\text{Cd}^{2+}$  inhibition shown by comparing the second pulse (in red) with the entire inhibition time course. (C) Delay of current inhibition upon  $\text{Cd}^{2+}$  application obtained at different voltages.  $[\text{Cd}^{2+}] = 300\text{ }\mu\text{M}$ . Solid curve is a voltage-dependent exponential fit for the relation between the delay time and the voltage, with an estimated electric distance of  $-0.025$ .  $n = 7$ . (D)  $[\text{Cd}^{2+}]$  dependence of the delay of current inhibition.  $V_m = -80\text{ mV}$ . Data points are the average of five to eight experiments (mean  $\pm$  SEM).

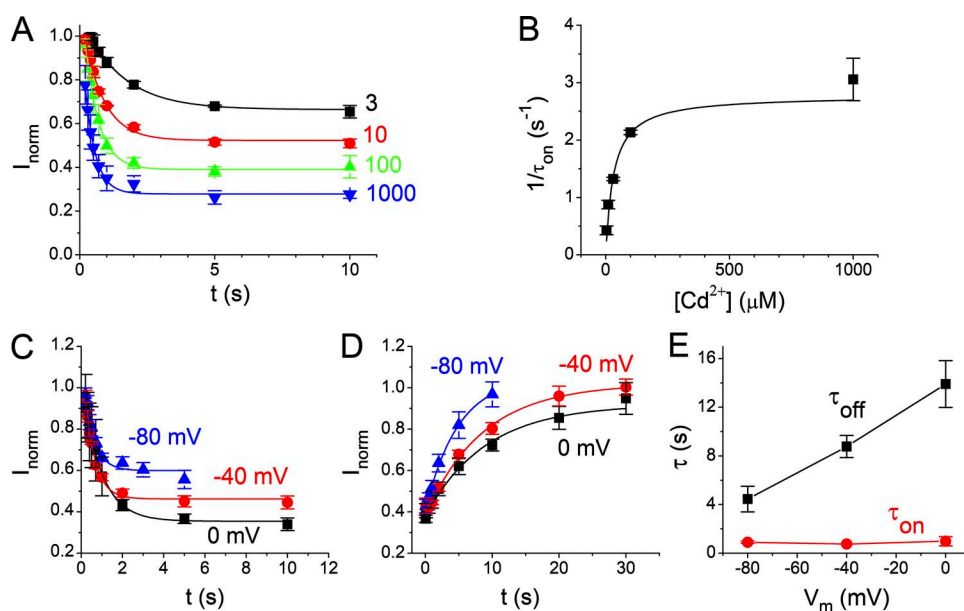
binding of  $\text{Cd}^{2+}$  to the channel;  $\text{Cd}^{2+}$  binding to the closed state of the channel is energetically more favored than the binding to the open state.

## DISCUSSION

Transition metal ions have been widely used to study the structure–function relationship of ion channels. In the *Shaker*  $\text{K}^+$  channel, for example, the state-dependent affinity change of extracellular  $\text{Cd}^{2+}$  inhibition revealed a structural change on the external mouth of the channel during the slow (C-type) inactivation (Yellen et al., 1994). Intracellularly applied  $\text{Cd}^{2+}$  was also used to show that the activation gate of the *Shaker*  $\text{K}^+$  channel can be trapped in the open state when an intersubunit metal bridge is formed (Holmgren et al., 1998). CLC-0 and CLC-1 have been found to be inhibited by  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$  applied from the extracellular side (Rychkov et al., 1997; Chen, 1998), and extracellular  $\text{Zn}^{2+}$  is known to inhibit WT CLC-0 by facilitating the closure of the slow gate (Chen, 1998). We found in the present study that the inhibition of WT CLC-0 by intracellular  $\text{Cd}^{2+}$  is likely mediated by a modulation of the slow gating of CLC-0 as well because intracellular  $\text{Cd}^{2+}$  has little effect on the fast gating of the channel (Fig. 2 C), and because the inhibition is largely suppressed by the C212S mutation (Fig. 2 D), which prevents the slow gate of CLC-0 from closing (Lin et al., 1999).

The apparent affinity of intracellular  $\text{Cd}^{2+}$  on WT CLC-0 is quite low—tens to hundreds of micromolars of  $[\text{Cd}^{2+}]$  is required for an observable effect. However, the potency of  $\text{Cd}^{2+}$  is significantly enhanced by tryptophan substitutions of I225 and V490, which correspond to dimer interface residues I201 and I422, respectively, in CLC-ec1. The I201W/I422W mutation in CLC-ec1 disrupts dimer architecture, leading to the formation of CLC monomers (Robertson et al., 2010). The corresponding mutations in CLC-0, however, do not separate the two channel subunits as indicated by a high FRET efficiency between the FRET donor and acceptor tagged to the C termini of the transmembrane domains (Fig. 3). The formation of a disulfide bond between the two cysteine residues at position 231 further demonstrates a dimeric CLC molecule in the I290W/V490W background mutations (Fig. 10). Despite the fact that CLC-0 with I225W/V490W mutations remains as a dimer, the mutant channel's voltage-dependent gating properties have been inverted from that of the WT channel (Fig. 4 A). It is conceivable that introducing bulky tryptophan side chains into the dimer interface alters the interaction between the two CLC subunits, thus increasing apparent affinity and kinetics of  $\text{Cd}^{2+}$  inhibition (Fig. 4 B), and facilitating disulfide bond formation between cysteines at position 231 (Fig. 10).

A closer examination of the  $\text{Cd}^{2+}$  inhibition on the I225W/V490W mutant reveals that intracellular  $\text{Cd}^{2+}$



**Figure 13.** Voltage dependence of the inhibition and recovery kinetics of the  $\text{Cd}^{2+}$  modulation of the I225W/V490W mutant of CLC-0. (A) Time course of current inhibition by various  $[\text{Cd}^{2+}]$  (in micromolars) at  $-40$  mV. (B) Inverse of the time constant of  $\text{Cd}^{2+}$  inhibition ( $k_{\text{on}} = 1/\tau_{\text{on}}$ ) as a function of  $[\text{Cd}^{2+}]$ . The values of  $\tau_{\text{on}}$  were obtained from single-exponential fits as in A. (C) Averaged time course for the inhibition by 30  $\mu\text{M}$   $\text{Cd}^{2+}$  at 0,  $-40$ , and  $-80$  mV. Data points were calculated by normalizing the current in various  $[\text{Cd}^{2+}]$  to the control current ( $[\text{Cd}^{2+}] = 0$ ). (D) Time course of the current recovery from  $\text{Cd}^{2+}$  inhibition. Data points were obtained by normalizing the current recovered after  $\text{Cd}^{2+}$  was washed out to that before the application of  $\text{Cd}^{2+}$ . (E) Time constants of the inhibition ( $\tau_{\text{on}}$ ) and recovery ( $\tau_{\text{off}}$ ) as a function of membrane voltage. Time constants were obtained by fitting the data points in C and D with single-exponential functions.



appears to have two effects. At low micromolar  $[Cd^{2+}]$ , the mutant channel's steady-state activation curve is shifted to more hyperpolarized voltages by  $Cd^{2+}$ . We observed a competition between intracellular  $Cd^{2+}$  inhibition and MTS modification (Fig. 6), a clear indication that like many other transition metal-binding proteins, the amino acid cysteine is directly involved in forming the  $Cd^{2+}$ -binding site. Further mutagenesis experiments showed that the mutation of C229S, H231Y, or both abolishes the  $Cd^{2+}$ -induced shift of the steady-state activation curve in WT CLC-0 and in the I225W/V490W mutant (Fig. 7), indicating that the high affinity  $Cd^{2+}$  inhibition is mediated through the binding site formed by C229 and H231. On the other hand, the maximal open probability of these mutants was still inhibited by  $Cd^{2+}$  at the millimolar concentrations, suggesting that the low affinity  $Cd^{2+}$  inhibition may be mediated by a different mechanism.

The residues C229 and H231 of CLC-0 correspond to R205 and Q207, respectively, in CLC-ec1, which are in close proximity to the same residues in the partner subunit (Fig. 1). As seen in many metalloproteins, tetrahedral is a preferred coordination arrangement for  $Cd^{2+}$  binding, usually involving four cysteine/histidine residues (Rulíšek and Vondrášek, 1998). Therefore, it is likely that the  $Cd^{2+}$  site in CLC-0 is formed by two subunits, each contributing two chelators from C229 and H231 to bind  $Cd^{2+}$ . These cysteine and histidine residues, however, are not conserved in CLC-1, thus explaining the fact that WT CLC-1 is insensitive to intracellular  $Cd^{2+}$ , as shown in Figs. 8 A and 9 A, and also reported previously by others (Rychkov et al., 1997; Fahlke et al., 1998). In this study, we are able to construct a  $Cd^{2+}$ -binding site in CLC-1 by engineering S294C/Y296H mutations to mimic the  $Cd^{2+}$  site in CLC-0. This strategy yields a CLC-1 mutant whose activity is modulated by low micromolar intracellular  $Cd^{2+}$ , although the consequence of the  $Cd^{2+}$  modulation is potentiation but not inhibition of the channel current! Such  $Cd^{2+}$  effect, again, is abolished by pretreating the channel with intracellular MTS reagents (Fig. 9 C), indicating that a  $Cd^{2+}$ -binding site is indeed created. Previous studies have indicated an interaction of the transmembrane domain and the C-terminal cytoplasmic domain of CLC proteins (Feng et al., 2010; Bennetts and Parker, 2013). Experiments in Fig. 11 show that removing the C-terminal region of CLC-0 changes the  $Cd^{2+}$  effect from inhibition to potentiation. Thus, the interaction of the transmembrane domain with the C-terminal cytoplasmic domain as well as the subunit interaction at the dimer interface are both involved in intracellular  $Cd^{2+}$  modulation of CLC-0.

These experimental results thus allow us to compare the functional roles of subunit interactions between CLC channels and bacterial CLC proteins. It was demonstrated previously that cross-linking the two CLC-ec1

subunits covalently poses little impact on the  $Cl^-$  transport function (Nguitrage and Miller, 2007). Similarly, the fast gating of CLC-0 and CLC-1 is not affected by restricting molecular motions at the dimer interface with  $Cd^{2+}$  (Figs. 2 and 9). This is consistent with the accepted concept that the  $Cl^-/H^+$  exchange cycle of CLC-ec1 and the fast gating of CLC channels are homologous, mediated by molecular motions (for example, swinging of the glutamate side chain) confined in individual subunits. Coordinating opening and closing of the two  $Cl^-$  pathways requires the slow/common gating mechanism that enables the cross talk of the two subunits through the dimer interface.  $Cd^{2+}$  binding or the introduction of bulky residues at the dimer interface would perturb such a cross talk and thus modulate slow/common gating of CLC channels. It is possible that mutation of I225W and V490W speeds up the inactivation of CLC-0 (closure of the slow gate), explaining the faster kinetics of  $Cd^{2+}$  inhibition in the I225W/V490W double mutant than in the WT channel. Interestingly,  $Cd^{2+}$  binding inhibits the current of WT CLC-0 and I225W/V490W but potentiates the current of CLC0-TM (Fig. 11) and the CLC-1 mutant S294C/Y296H (Figs. 8 and 9). The different consequences of  $Cd^{2+}$  binding (potentiation vs. inhibition of the current) may result from the difference between the cross talk of the two subunits in these various channels. Another possibility is that the allosteric interactions between the  $Cd^{2+}$ -binding site and the C-terminal cytoplasmic domain are different in these channels. It will await further studies to understand the different functional consequences of  $Cd^{2+}$  modulations in CLC-0, CLC-1, and the C terminus-truncated CLC channel.

If C229 and H231 of CLC-0 are located at the intracellular end of the transmembrane domain, like their corresponding residues R205 and Q207 in CLC-ec1 (Fig. 1), it is expected that the  $Cd^{2+}$  site would be outside the transmembrane electric field, and thus  $Cd^{2+}$  binding should be voltage independent. Indeed, the apparent on-rate of  $Cd^{2+}$  is voltage independent (Fig. 13 E). However, the apparent  $Cd^{2+}$  affinity is lower at more hyperpolarized voltages (Fig. 5 C) because membrane hyperpolarization (which favors the opening of the I225W/V490W mutant) accelerates  $Cd^{2+}$  dissociation (Fig. 13, D and E). The voltage dependence of the apparent off-rate thus suggests a state-dependent  $Cd^{2+}$  inhibition; namely,  $Cd^{2+}$  binding to the open state is energetically less favorable than the binding to the closed state. Furthermore,  $Cd^{2+}$  inhibition shows an appreciable delay (Fig. 12) and the apparent on-rate of  $Cd^{2+}$  inhibition is a saturable function of  $[Cd^{2+}]$  (Fig. 13 B), both corroborating the idea that  $Cd^{2+}$  binding to the open-state channel cannot immediately shut the pore. Instead, it will require a subsequent molecular event, likely a conformational change associated with slow gating, to close the channel.

In summary, we have characterized the inhibition of CLC-0's slow gating by intracellular  $\text{Cd}^{2+}$ , and have identified a  $\text{Cd}^{2+}$ -binding site formed by C229 and H231 at the intracellular end of the dimer interface of the transmembrane domain. We have also shown that the residues I225 and V490 of CLC-0, like the corresponding residues in other CLC channels, are important for the channel gating mechanism likely because of their roles in the subunit interaction. Binding of  $\text{Cd}^{2+}$  to the dimer interface and mutations of dimer interface residues thus may modulate the slow/common gating of CLC channels by altering subunit interactions.

We would like to thank Drs. Robert Fairclough and Tzyh-Chang Hwang for critical readings of the manuscript.

This work was supported by a grant from National Institutes of Health (R01GM065447) and by the Jong L. Chen Family Neuroscience Research Fund.

The authors declare no competing financial interests.

Merritt C. Maduke served as editor.

Submitted: 17 April 2015

Accepted: 9 November 2015

## REFERENCES

- Accardi, A., and M. Pusch. 2000. Fast and slow gating relaxations in the muscle chloride channel CLC-1. *J. Gen. Physiol.* 116:433–444. <http://dx.doi.org/10.1085/jgp.116.3.433>
- Bennetts, B., and M.W. Parker. 2013. Molecular determinants of common gating of a ClC chloride channel. *Nat. Commun.* 4:2507. <http://dx.doi.org/10.1038/ncomms3507>
- Bennetts, B., G.Y. Rychkov, H.L. Ng, C.J. Morton, D. Stapleton, M.W. Parker, and B.A. Cromer. 2005. Cytoplasmic ATP-sensing domains regulate gating of skeletal muscle ClC-1 chloride channels. *J. Biol. Chem.* 280:32452–32458. <http://dx.doi.org/10.1074/jbc.M502890200>
- Bykova, E.A., X.D. Zhang, T.Y. Chen, and J. Zheng. 2006. Large movement in the C terminus of CLC-0 chloride channel during slow gating. *Nat. Struct. Mol. Biol.* 13:1115–1119. <http://dx.doi.org/10.1038/nsmb1176>
- Chen, T.Y. 1998. Extracellular zinc ion inhibits ClC-0 chloride channels by facilitating slow gating. *J. Gen. Physiol.* 112:715–726. <http://dx.doi.org/10.1085/jgp.112.6.715>
- Chen, T.Y. 2005. Structure and function of clc channels. *Annu. Rev. Physiol.* 67:809–839. <http://dx.doi.org/10.1146/annurev.physiol.67.032003.153012>
- Duffield, M., G. Rychkov, A. Bretag, and M. Roberts. 2003. Involvement of helices at the dimer interface in ClC-1 common gating. *J. Gen. Physiol.* 121:149–161. <http://dx.doi.org/10.1085/jgp.20028741>
- Dutzler, R., E.B. Campbell, M. Cadene, B.T. Chait, and R. MacKinnon. 2002. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature.* 415:287–294. <http://dx.doi.org/10.1038/415287a>
- Dutzler, R., E.B. Campbell, and R. MacKinnon. 2003. Gating the selectivity filter in ClC chloride channels. *Science.* 300:108–112. <http://dx.doi.org/10.1126/science.1082708>
- Erickson, M.G., B.A. Alseikhan, B.Z. Peterson, and D.T. Yue. 2001. Preassociation of calmodulin with voltage-gated  $\text{Ca}^{2+}$  channels revealed by FRET in single living cells. *Neuron.* 31:973–985.
- Estévez, R., and T.J. Jentsch. 2002. CLC chloride channels: correlating structure with function. *Curr. Opin. Struct. Biol.* 12:531–539. [http://dx.doi.org/10.1016/S0959-440X\(02\)00358-5](http://dx.doi.org/10.1016/S0959-440X(02)00358-5)
- Fahlke, C., T.H. Rhodes, R.R. Desai, and A.L. George Jr. 1998. Pore stoichiometry of a voltage-gated chloride channel. *Nature.* 394:687–690. <http://dx.doi.org/10.1038/29319>
- Feng, L., E.B. Campbell, Y. Hsiung, and R. MacKinnon. 2010. Structure of a eukaryotic CLC transporter defines an intermediate state in the transport cycle. *Science.* 330:635–641. <http://dx.doi.org/10.1126/science.1195230>
- Holmgren, M., K.S. Shin, and G. Yellen. 1998. The activation gate of a voltage-gated  $\text{K}^{+}$  channel can be trapped in the open state by an intersubunit metal bridge. *Neuron.* 21:617–621. [http://dx.doi.org/10.1016/S0896-6273\(00\)80571-1](http://dx.doi.org/10.1016/S0896-6273(00)80571-1)
- Jentsch, T.J. 2008. CLC chloride channels and transporters: From genes to protein structure, pathology and physiology. *Crit. Rev. Biochem. Mol. Biol.* 43:3–36. <http://dx.doi.org/10.1080/10409230701829110>
- Jentsch, T.J., I. Neagoe, and O. Scheel. 2005. CLC chloride channels and transporters. *Curr. Opin. Neurobiol.* 15:319–325. <http://dx.doi.org/10.1016/j.conb.2005.05.002>
- Kubisch, C., T. Schmidt-Rose, B. Fontaine, A.H. Bretag, and T.J. Jentsch. 1998. ClC-1 chloride channel mutations in myotonia congenita: Variable penetrance of mutations shifting the voltage dependence. *Hum. Mol. Genet.* 7:1753–1760. <http://dx.doi.org/10.1093/hmg/7.11.1753>
- Lin, C.W., and T.Y. Chen. 2003. Probing the pore of ClC-0 by substituted cysteine accessibility method using methane thio-sulfonate reagents. *J. Gen. Physiol.* 122:147–159. <http://dx.doi.org/10.1085/jgp.200308845>
- Lin, Y.W., C.W. Lin, and T.Y. Chen. 1999. Elimination of the slow gating of ClC-0 chloride channel by a point mutation. *J. Gen. Physiol.* 114:1–12. <http://dx.doi.org/10.1085/jgp.114.1.1>
- Ludewig, U., M. Pusch, and T.J. Jentsch. 1996. Two physically distinct pores in the dimeric ClC-0 chloride channel. *Nature.* 383:340–343. <http://dx.doi.org/10.1038/383340a0>
- Maduke, M., C. Miller, and J.A. Mindell. 2000. A decade of CLC chloride channels: Structure, mechanism, and many unsettled questions. *Annu. Rev. Biophys. Biomol. Struct.* 29:411–438. <http://dx.doi.org/10.1146/annurev.biophys.29.1.411>
- Middleton, R.E., D.J. Pheasant, and C. Miller. 1994. Purification, reconstitution, and subunit composition of a voltage-gated chloride channel from *Torpedo* electroplax. *Biochemistry.* 33:13189–13198. <http://dx.doi.org/10.1021/bi00249a005>
- Middleton, R.E., D.J. Pheasant, and C. Miller. 1996. Homodimeric architecture of a ClC-type chloride ion channel. *Nature.* 383:337–340. <http://dx.doi.org/10.1038/383337a0>
- Miller, C. 1982. Open-state substructure of single chloride channels from *Torpedo* electroplax. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:401–411. <http://dx.doi.org/10.1098/rstb.1982.0140>
- Miller, C., and M.M. White. 1984. Dimeric structure of single chloride channels from *Torpedo* electroplax. *Proc. Natl. Acad. Sci. USA.* 81:2772–2775. <http://dx.doi.org/10.1073/pnas.81.9.2772>
- Miyawaki, A., and R.Y. Tsien. 2000. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* 327:472–500.
- Nguiragool, W., and C. Miller. 2007. CLC  $\text{Cl}^{-}/\text{H}^{+}$  transporters constrained by covalent cross-linking. *Proc. Natl. Acad. Sci. USA.* 104:20659–20665. <http://dx.doi.org/10.1073/pnas.0708639104>
- Puljung, M.C., and W.N. Zagotta. 2011. Labeling of specific cysteines in proteins using reversible metal protection. *Biophys. J.* 100:2513–2521. <http://dx.doi.org/10.1016/j.bpj.2011.03.063>
- Pusch, M., K. Steinmeyer, M.C. Koch, and T.J. Jentsch. 1995. Mutations in dominant human myotonia congenita drastically alter the voltage dependence of the ClC-1 chloride channel. *Neuron.* 15:1455–1463. [http://dx.doi.org/10.1016/0896-6273\(95\)90023-3](http://dx.doi.org/10.1016/0896-6273(95)90023-3)

- Robertson, J.L., L. Kolmakova-Partensky, and C. Miller. 2010. Design, function and structure of a monomeric ClC transporter. *Nature*. 468:844–847. <http://dx.doi.org/10.1038/nature09556>
- Rulišek, L., and J. Vondrášek. 1998. Coordination geometries of selected transition metal ions ( $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Hg}^{2+}$ ) in metalloproteins. *J. Inorg. Biochem.* 71:115–127. [http://dx.doi.org/10.1016/S0162-0134\(98\)10042-9](http://dx.doi.org/10.1016/S0162-0134(98)10042-9)
- Rychkov, G.Y., D. St J. Astill, B. Bennetts, B.P. Hughes, A.H. Bretag, and M.L. Roberts. 1997. pH-dependent interactions of  $\text{Cd}^{2+}$  and a carboxylate blocker with the rat ClC-1 chloride channel and its R304E mutant in the Sf-9 insect cell line. *J. Physiol.* 501:355–362. <http://dx.doi.org/10.1111/j.1469-7793.1997.355bn.x>
- Saviane, C., F. Conti, and M. Pusch. 1999. The muscle chloride channel ClC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *J. Gen. Physiol.* 113:457–468. <http://dx.doi.org/10.1085/jgp.113.3.457>
- Stauber, T., S. Weinert, and T.J. Jentsch. 2012. Cell biology and physiology of CLC chloride channels and transporters. *Compr. Physiol.* 2:1701–1744.
- Tseng, P.Y., B. Bennetts, and T.Y. Chen. 2007. Cytoplasmic ATP inhibition of CLC-1 is enhanced by low pH. *J. Gen. Physiol.* 130:217–221. <http://dx.doi.org/10.1085/jgp.200709817>
- Yellen, G., D. Sodickson, T.Y. Chen, and M.E. Jurman. 1994. An engineered cysteine in the external mouth of a  $\text{K}^+$  channel allows inactivation to be modulated by metal binding. *Biophys. J.* 66:1068–1075. [http://dx.doi.org/10.1016/S0006-3495\(94\)80888-4](http://dx.doi.org/10.1016/S0006-3495(94)80888-4)
- Zhang, X.D., and T.Y. Chen. 2009. Amphiphilic blockers punch through a mutant CLC-0 pore. *J. Gen. Physiol.* 133:59–68. <http://dx.doi.org/10.1085/jgp.200810005>
- Zhang, X.D., Y. Li, W.P. Yu, and T.Y. Chen. 2006. Roles of K149, G352, and H401 in the channel functions of CLC-0: Testing the predictions from theoretical calculations. *J. Gen. Physiol.* 127:435–447. <http://dx.doi.org/10.1085/jgp.200509460>
- Zhang, X.D., P.Y. Tseng, and T.Y. Chen. 2008. ATP inhibition of CLC-1 is controlled by oxidation and reduction. *J. Gen. Physiol.* 132:421–428. <http://dx.doi.org/10.1085/jgp.200810023>
- Zhang, X.D., W.P. Yu, and T.Y. Chen. 2010. Accessibility of the CLC-0 pore to charged methanethiosulfonate reagents. *Biophys. J.* 98:377–385. <http://dx.doi.org/10.1016/j.bpj.2009.09.066>
- Zheng, J., M.C. Trudeau, and W.N. Zagotta. 2002. Rod cyclic nucleotide-gated channels have a stoichiometry of three CNGA1 subunits and one CNGB1 subunit. *Neuron*. 36:891–896.
- Zheng, J., M.D. Varnum, and W.N. Zagotta. 2003. Disruption of an intersubunit interaction underlies  $\text{Ca}^{2+}$ -calmodulin modulation of cyclic nucleotide-gated channels. *J. Neurosci.* 23:8167–8175.